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RESEARCH ARTICLE

Activation of a peroxisomal *Pichia pastoris* D-amino acid oxidase, which uses D-alanine as a preferred substrate, depends on pyruvate carboxylase

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Keywords

D-amino acid oxidase; peroxisome; pyruvate carboxylase; *Pichia pastoris*; moonlighting.

Abstract

D-Amino acid oxidase (DAO) is an important flavo-enzyme that catalyzes the oxidative deamination of D-amino acids into the corresponding α -keto acid, ammonia and H₂O₂. We identified two amino acid oxidases in the methylotrophic yeast *Pichia pastoris*: Dao1p, which preferentially uses D-alanine as a substrate, and Dao2p, which uses D-aspartate as a preferred substrate. Dao1p has a molecular mass of 38.2 kDa and a pH optimum of 9.6. This enzyme was localized to peroxisomes, albeit a typical peroxisomal targeting signal is lacking. Interestingly, *P. pastoris* mutant strains, defective in the enzyme pyruvate carboxylase, showed a pronounced growth defect on D-alanine, concomitant with a significant reduction in Dao1p activity relative to the wild-type control. This indicates that pyruvate carboxylase functions in import and/or activation of *P. pastoris* Dao1p.

Introduction

D-Amino acid oxidase (DAO, EC 1.4.3.3) was first described in 1935, and since then, has become a model enzyme for the dehydrogenase-oxidase family of flavoproteins (Krebs, 1935). It catalyzes the oxidative deamination of D-amino acids to the corresponding α -keto acid with a strict stereospecificity. A noncovalently bound FAD moiety is used as an electron acceptor, which reoxidizes via molecular oxygen to produce H₂O₂. DAO is predominantly observed in eukaryotes; however, one bacterial DAO was characterized recently (Geueke *et al.*, 2007).

Industrially relevant applications for DAO are the production of α -keto acids and pure L-amino acids, the analytical determination of D-amino acids and especially the production of the cephalosporin antibiotics precursor 7-aminocephalosporanic acid. More than 50 different semi-synthetic cephalosporins are synthesized from this antibiotic agent by modification of the side chain (reviewed in Pollegioni *et al.*, 2008).

DAO contains a peroxisomal targeting signal 1 (PTS1) in various eukaryote organisms and is imported into peroxisomes by the soluble receptor Pex5p (Yurimoto *et al.*, 2000). Peroxisomes are ubiquitous and single-membrane-bound organelles. The physiological function, protein composition and abundance of peroxisomes strongly depend on the organism or the cell type in which they occur and may alter with changes in environmental conditions (van der Klei & Veenhuis, 2006). The majority of peroxisomal matrix proteins are imported via the PTS1. The signal consists of a tri-peptide at the extreme C-terminus with the consensus sequence (S/A/C)-(K/R/H)-L, and is recognized by the soluble import receptor Pex5p (Swinkels *et al.*, 1992; McCollum *et al.*, 1993). However, Pex5p is also required for import of the so-called 'non-PTS' proteins that either lack a recognizable targeting signal or contain a signal that is redundant for import (Klein *et al.*, 2002; Gunkel *et al.*, 2004).

In methylotrophic yeast, the activation of another member of the dehydrogenase-oxidase family, alcohol oxidase

(AO), depends on the chaperone-like activity of the cytosolic protein pyruvate carboxylase (Pyc1p) (Ozimek *et al.*, 2003). Pyc1p replenishes the citric acid cycle with oxaloacetate, but contains an additional (moonlighting) function in the activation pathway of AO that is independent of its enzyme activity. Like DAO, AO also is a peroxisomal and flavin-containing enzyme. In *pyc1*-deficient strains of *Hansenula polymorpha* and *Pichia pastoris*, AO is mislocalized to the cytosol as a monomer that lacks FAD.

Here, we report the presence of two DAO homologs in the yeast *P. pastoris*: Dao1p, which preferentially uses D-alanine as a substrate, and Dao2p, which has D-aspartate as a preferred substrate.

Detailed analysis of Dao1p revealed that it has a pH optimum of 9.6. This enzyme was localized to peroxisomes despite the absence of a typical peroxisomal targeting signal. Interestingly, cells of a pyruvate carboxylase-deficient strain showed a growth defect on media containing D-alanine and reduced activities of Dao1p. This suggests that pyruvate carboxylase is involved in Dao1p import and/or activation, like it is required for the import and activation of the peroxisomal flavo-enzyme AO (Ozimek *et al.*, 2003).

Materials and methods

Organisms and culture conditions

The *P. pastoris* strains used in this study are listed in Table 1. All strains were cultured at 30 °C. The media components were as follows: solid media contained 0.17% yeast nitrogen base, 2.2% agar, pH 6.0, with either 0.5% glucose or 0.3% ethanol in the presence of 0.5% ammonium sulfate. Oleate plates contained 0.1% oleic acid, 0.5% Tween 80, 0.1% yeast extract, 0.5% ammonium sulfate, 0.17% yeast nitrogen base and 2.2% agar, pH 6.0. D-Alanine plates contained 0.6% D-alanine, 0.5% glucose, 0.17% yeast nitrogen base and 2.2% agar, pH 6.0. Liquid mineral medium (van Dijken *et al.*, 1976) was supplemented with 0.5% glucose and 0.25% ammonium sulfate or 0.5% methanol with either 0.25% ammonium sulfate or 0.25% D-alanine. Histidine, arginine and L-aspartate (60 mg L⁻¹) were added when needed.

Table 1. *Pichia pastoris* strains used in this study

Strain	Reference
MP36 <i>his3</i>	Menendez (1998)
MP36 <i>his3</i> , <i>pyc1::HIS3</i>	Menendez (1998)
PPY12 <i>his4</i> , <i>arg4</i>	Gould (1992)
PPY12 <i>his4</i> , <i>arg4</i> , RPPA05600A::zeocin	This study
PPY12 <i>his4</i> , <i>arg4</i> , RPPA06337A::hygromycin	This study
PPY12 <i>his4</i> , <i>arg4</i> , <i>HIS4::pPIC3.5K-DAO</i>	This study

Identification of PpDAO

A BLAST search of the *P. pastoris* genome database (Integrated Genomics, Chicago) revealed two homologs, RPPA05600 and RPPA06337, of human D-amino acid oxidase (HsDAO). Based on homology to known genes and its function (see Results), RPPA05600 was designated as *PpDAO1*.

Construction of deletion strains

The 0.2 kbp 5' flanking region of RPPA05600 was amplified from PPY12 genomic DNA using the primers SAK50 and SAK51 (Table 2). The 0.2 kbp 3' flanking region of RPPA05600 was amplified using oligos SAK52 and SAK53. Both fragments were cloned into pBS-zeo, to obtain pBS-ΔRPPA05600. The plasmid pBS-zeo was constructed in the following way: the 1.1 kbp zeocin cassette was amplified from pREMI-Z (van Dijk *et al.*, 2001) using the primers Zeo-XbaI-FW and Zeo-XhoI-RV and cloned into the XbaI/XhoI fragment of pBluescript II KS+ (Stratagene, La Jolla, CA). The deletion cassette was amplified from pBS-ΔRPPA05600 with SAK50 and SAK53 and used to transform PPY12. Transformants were selected for their ability to grow in the presence of zeocin and checked by Southern blotting.

The 0.4 kbp 5' flanking region of RPPA06337 was amplified from PPY12 genomic DNA with the primers SAK54 and SAK55. The 0.2 kbp 3' flanking region of RPPA06337 was amplified using oligos SAK56 and SAK57. Both fragments were cloned into pBS-del-PEX3-hph to obtain pBS-ΔRPPA06337. pBS-del-PEX3-hph was constructed in the following way: the SacI site of pAG32 (Goldstein & McCusker, 1999) was removed by digestion, T4 DNA polymerase blunt ending and relegation, yielding pAG32B. In the second step, a 1.7 kbp BglII/EcoRV fragment of pAG32B was ligated to a 3.6 kbp BglII/EcoRV vector fragment of pRBG3 (Baerends *et al.*, 2000). The deletion cassette was amplified from pBS-ΔRPPA06337 with SAK54 and SAK57 and used to transform PPY12. Transformants were selected for their

Table 2. Oligonucleotides used in this study

Primer	Sequence (5' → 3')
SAK50	GGGGAGCTCATCTCTAAATCCCCACTG
SAK51	CCAAGCGCCGCTTCTTGCCAGTGTTCAGAG
SAK52	GGCTCGAGCTCCAACAGATAGCTCATAC
SAK53	GGTGAAGGTACCTTACAAGACGACAGTGCCG
SAK54	GGATGTCGACCTTGCGGTGTCGCTACTTC
SAK55	GCGAGATCTGTTGGGATGACCTCTCTC
SAK56	GAGGATCCGCATCTCTGCTGTCTGTC
SAK57	CAGAGCTCGCTCGCTGAAATTTGTGG
SAK94.1	CGCGGATCCATGACTGATTCCAATACGTTATCATTGG
SAK95.1	GCAGGCGGCCGCTCATTTGGCAGCTTCAAATAC
Zeo-XbaI-FW	CGCTCTAGAGTCATAGCTGTTCCGATCCC
Zeo-XhoI-RV	CGCCTCGAGGCTCACATGTTGCTCTCCAGC

ability to grow in the presence of hygromycin and checked by Southern blotting.

Ectopic expression

The full-length *DAO1* ORF was amplified from PPY12 genomic DNA using the primers SAK94.1 and SAK95.1 and expressed from the AOX promoter in the vector pPIC3.5K (Invitrogen, Breda, the Netherlands). The resulting plasmid, designated pPIC3.5K-DAO, was linearized with *SalI* and transformed to PPY12 cells. Transformants were selected by histidine prototrophy and confirmed by Southern blotting.

Molecular techniques

The oligonucleotides used in this study are presented in Table 2. All standard DNA procedures were performed as described (Sambrook *et al.*, 1989). Electro-transformation was performed according to Faber *et al.* (1994).

Biochemical methods

DAO- and D-aspartate oxidase-specific enzyme activities were measured spectrophotometrically at 30 °C in crude cell extracts essentially as described previously (Sulter *et al.*, 1990). FAD (5 µM) was added during the reaction, and 50 mM D-alanine and 10 mM D-aspartate, respectively, were used as a substrate. Protein concentrations were determined using the Bio-Rad assay kit and bovine serum albumin as a standard (Bio-Rad GmbH, Munich, Germany). For determination of the pH optimum, 100 mM sodium carbonate–bicarbonate buffer was utilized that buffers well between pH 8.5 and 11.5.

Electron microscopy

Protoplasts were generated essentially as described by Faber *et al.* (1998), with the exception that cells were treated with 10 mM DTT before protoplasting was performed in the presence of 2.4 M sorbitol. Fixation, cytochemical staining and electron microscopy of protoplasts were performed according to Veenhuis *et al.* (1976).

Results

Identification of two DAOs in *P. pastoris*

In order to search for DAO homologs of *P. pastoris*, the gene coding for HsDAO was used as a query against the *P. pastoris* genome database. Two genes, RPPA05600 and RPPA06337, which code for two proteins of 344 and 404 amino acids, respectively, were identified. RPPA05600 was designated *DAO1* and RPPA06337 was named *DAO2*. A database search revealed that the protein product of *DAO1*, Dao1p, shows the highest homology to *Candida boidinii* DAO (66%

identity). Dao2p is 38% identical to CbDAO (Fig. 1). Both proteins contain the N-terminal GxGxxG nucleotide-binding sequence that relates to the presence of a Rossman fold in the protein structure, a common structural feature of flavin-containing oxidoreductases. Neither Dao1p nor Dao2p contains a peroxisomal targeting signal type 1 or 2.

Dao1p exhibits D-alanine oxidase activity

DAO1 and *DAO2* deletion strains were constructed in the *P. pastoris* PPY12 background and analyzed for defects in the utilization of D-alanine (Fig. 2) using a spot assay. Serial dilutions of cells were spotted on YND plates containing glucose in the presence of D-alanine as the sole nitrogen source. The data presented in Fig. 2a show that cells of the *Δdao1* strain display a strong growth defect, relative to *Δdao2* and the wild-type (WT) (PPY12) control. This growth defect was accompanied by a significant reduction in the D-alanine oxidase-specific enzyme activity, detected in crude extracts prepared from D-alanine-grown *Dao1p*-deficient cells (Fig. 2b). Identically grown cells of the *Δdao2* strain contained normal DAO activities for D-alanine comparable to those in WT controls. To confirm that the D-alanine oxidase activity is exhibited by the product of the *DAO1* gene, *DAO1* was overexpressed under control of the strong AOX promoter. Cells of the transformants were grown on mineral media supplemented with methanol as the sole carbon source to induce the synthesis of Dao1p. The D-alanine oxidase-specific activities detected in crude cell extracts of these cells amounted to ~2.3 U mg⁻¹ protein, which is about 120 times higher than the endogenous WT activities.

The pH optimum of the enzyme was determined using crude extracts of Dao1p-overproducing cells (Fig. 3). The enzyme showed a pH optimum of pH=9.6. The pH optimum appeared to be rather broad and the enzyme functions well between pH 9.1 and 10.6.

Subsequently, peptide antibodies were generated against Dao1p. These antibodies recognized a specific protein band in Western blots, prepared from crude extracts of the *DAO1* overexpression strain that was absent in the *Δdao1* strain (data not shown). However, in PPY12 WT cells, no protein band was detected. From this, we conclude that Dao1p is present at low levels in WT cells, consistent with the relatively low specific enzyme activities.

Dao2p exhibits D-aspartate oxidase activity

Dao1p and Dao2p share approximately 35% sequence identity with human D-aspartate oxidase. To determine whether one of the proteins displays D-aspartate oxidase activity, the WT strain PPY12 and the *Δdao1* and *Δdao2* strains were grown on glucose in the presence of D-aspartate as the sole nitrogen source (Fig. 4). Enzyme measurements

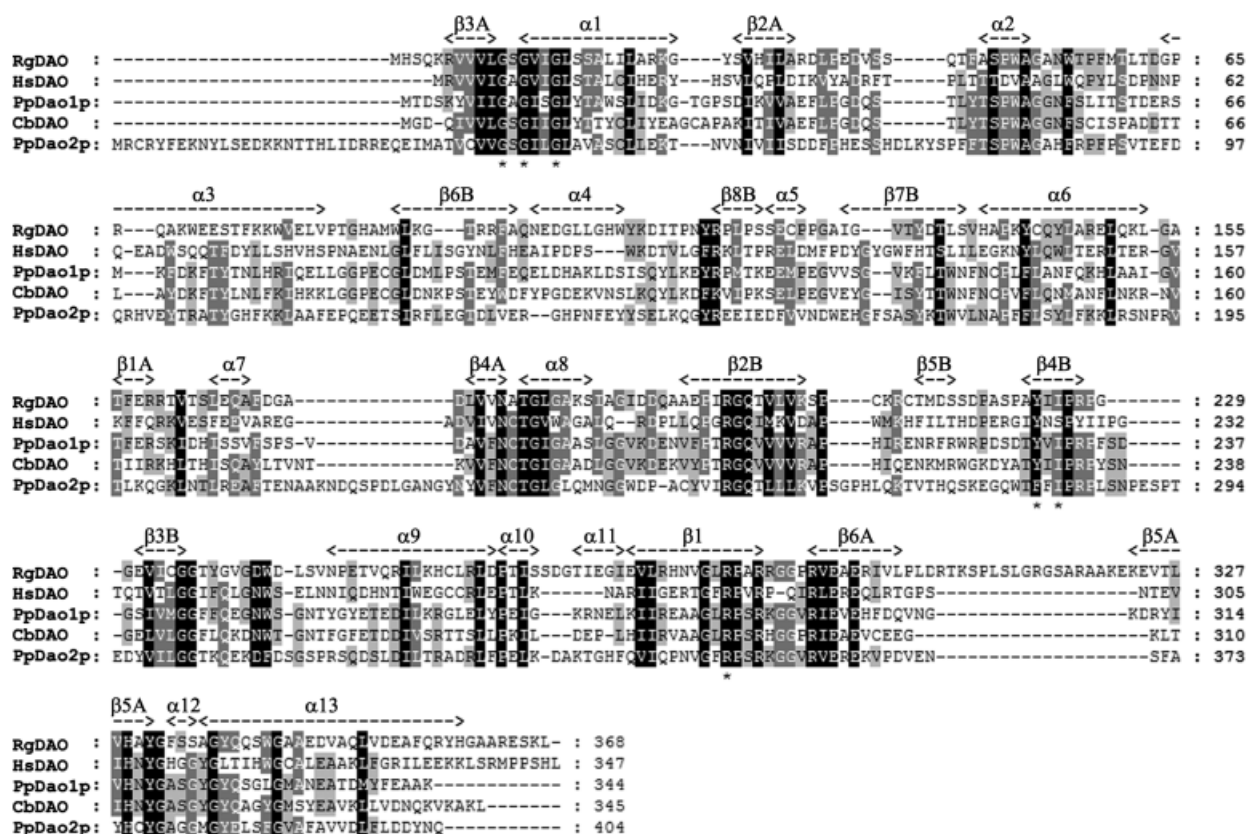


Fig. 1. Sequence alignment of DAOs from *Rhodotorula gracilis*, *Homo sapiens*, *Candida boidinii* and *Pichia pastoris*. Catalytically important residues are marked with an asterisk. Secondary structural elements from *R. gracilis* are depicted above the alignment (α, α helix; β, β strand).

using crude extracts of these cells demonstrated that the WT host and *Δdao1* cells contained comparable amounts of D-aspartate oxidase activity, whereas the enzyme activity was below the limit of detection in extracts of *Δdao2* cells.

Dao1p is localized to peroxisomes

All DAOs identified so far contain a C-terminal PTS1 and have been localized to peroxisomes (Perotti *et al.*, 1987; Perotti *et al.*, 1991). The C-terminal residues of Dao1p, -AAK, do not match the PTS1 consensus. Accordingly, neither a PTS1 (Neuberger *et al.*, 2003) nor the PTS2 predictor (Schlueter *et al.*, 2007) recognized the presence of a known PTS in Dao1p. As the low Dao1p levels did not allow detection by immunocytochemistry using the anti-Dao1p antibodies, we aimed to determine the subcellular location of the enzyme using the very sensitive cerium-based cytochemical method (Veenhuis *et al.*, 1976). This method is based on the trapping of the H₂O₂ side product generated by the enzyme at the site of its location. Incubation of protoplasts prepared from cells of the WT (PPY12) strain in the presence of CeCl₃ and D-alanine resulted in a cerium

reaction product that was confined to the peroxisomal matrix (Fig. 5). This convincingly demonstrates that in WT cells, Dao1p activity is confined to the peroxisomal matrix.

Growth on D-alanine and Dao1p enzyme activity are decreased in a *Δpyc1* strain

Previous reports showed that pyruvate carboxylase (Pyc1p) is required for the import and assembly of the peroxisomal flavo-enzyme AO in the yeasts *H. polymorpha* and *P. pastoris* (Ozimek *et al.*, 2003). This led us to investigate whether Pyc1p was also required for Dao1p activation. First, we analyzed the growth properties of a *P. pastoris* *PYC1* deletion strain (*Δpyc1*) on various carbon and nitrogen sources. The data depicted in Fig. 6a show that a strong reduction in growth was observed for *Δpyc1* on glucose/D-alanine. However, *Δpyc1* grew like the WT host on glucose/(NH₄)₂SO₄, ethanol/(NH₄)₂SO₄ and oleate/(NH₄)₂SO₄.

Pyc1p is an anaplerotic enzyme that replenishes the tricarboxylic acid cycle (TCA cycle) with oxaloacetate from pyruvate. This implies that *Δpyc1* cells of *P. pastoris* have to be supplemented with aspartate or glutamate to facilitate

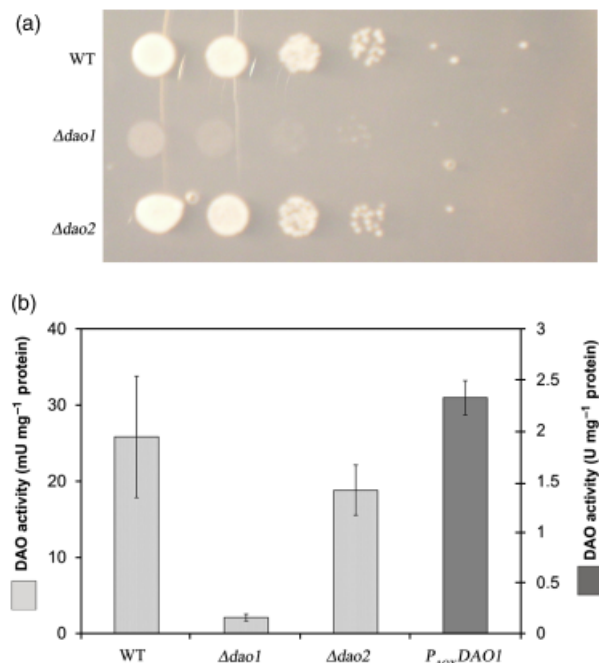


Fig. 2. Dao1p exhibits DAO activity. (a) Spot assay of *Pichia pastoris* WT (PPY12), $\Delta dao1$ and $\Delta dao2$ on solid media containing glucose and 0.6% D-alanine. Cells were grown for 3 days at 30 °C. (b) Dao1p-specific enzyme activities, determined in crude extracts of glucose/D-alanine cultured cells. The activities in extracts of $\Delta dao1$ -deficient cells are strongly decreased. Overexpression of *DAO1* under control of the inducible *AOX* promoter increases Dao1p enzyme activities to 2.3 U mg⁻¹ protein. The mean value of two independent experiments is shown.

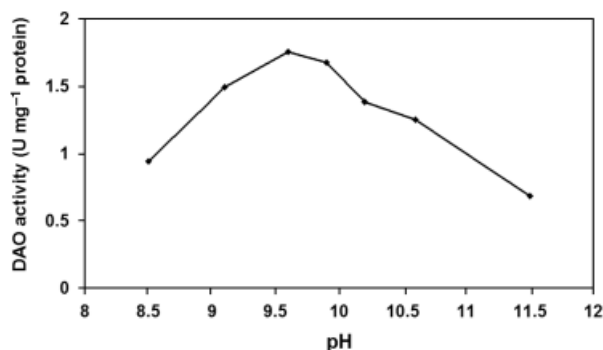


Fig. 3. Dao1p has an alkaline pH optimum. The specific activity of Dao1p was determined using extracts of WT PPY12 cells that overexpress *DAO1*. Dao1p displays the highest activity between pH 9.5 and 10.0.

growth on glucose in liquid media (Stucka *et al.*, 1991; Brewster *et al.*, 1994). On ethanol, however, cells grow normally due to the induction of a bypass pathway, the glyoxylate cycle, which complements for the withdrawal of intermediates from the TCA cycle. Therefore, additional growth experiments were performed in ethanol-containing liquid media supplemented with various nitrogen sources to

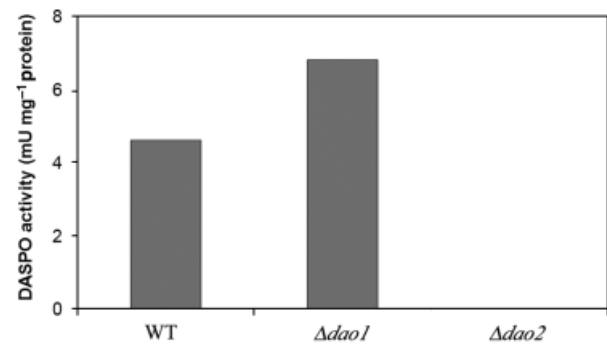


Fig. 4. Dao2p exhibits D-aspartate oxidase activity. Specific D-aspartate oxidase activities were determined in crude extracts of glucose/D-alanine cultured cells (WT PPY12). $\Delta dao2$ shows no activity toward D-aspartate, whereas in $\Delta dao1$ cells, the activities toward D-aspartate are comparable to those in WT cells.

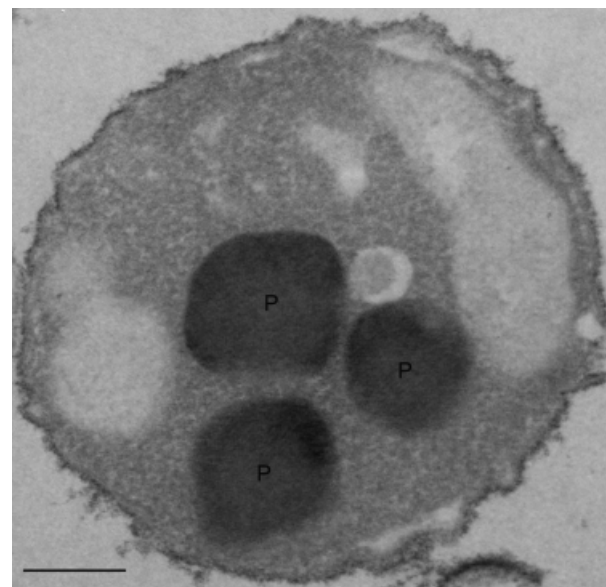


Fig. 5. Dao1p is a peroxisomal enzyme. After incubation of protoplasts (WT PPY12) in the presence of CeCl₃ and D-alanine, the cerous reaction product was confined to peroxisomes, demonstrating that Dao1p is a component of the peroxisome lumen. P, peroxisome. The scale bar represents 0.5 μm.

assess whether the growth defect of a $\Delta pyc1$ strain on D-alanine was specific for this nitrogen source (Fig. 6b). $\Delta pyc1$ cells grew like WT on ethanol/(NH₄)₂SO₄ (data not shown) and ethanol/L-aspartate. However, the growth of $\Delta pyc1$ cells on ethanol/D-alanine is retarded, consistent with the growth data obtained on solid plates.

The observed defect of $\Delta pyc1$ to grow on ethanol/D-alanine was associated with a strong decrease (approximately 70%) in Dao1p-specific enzyme activity relative to the activity detected in the WT host strain (Fig. 6c). This

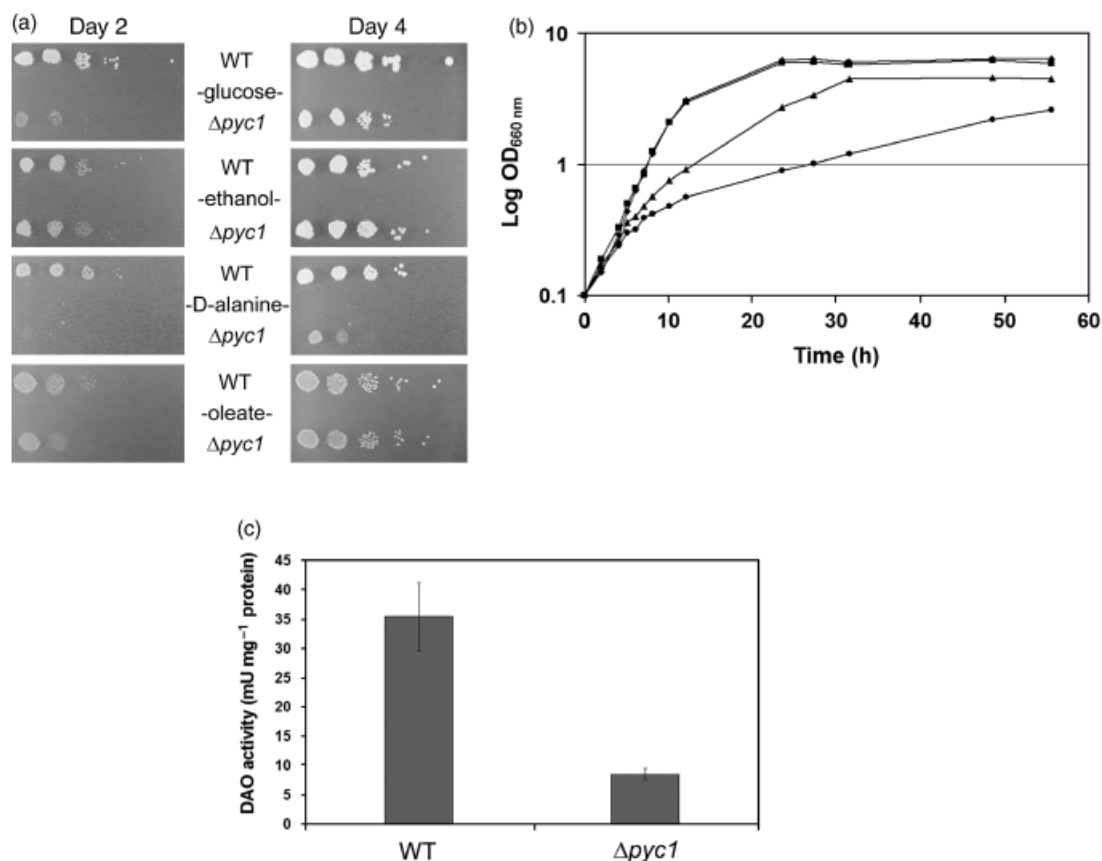


Fig. 6. Growth on D-alanine and Dao1p enzyme activities are decreased in *Pichia pastoris* $\Delta pyc1$ cells. (a) Spot assay of WT (MP36) and the $\Delta pyc1$ cells derived from this strain on agar plates supplemented with, respectively, glucose/ $\text{NH}_4(\text{SO}_4)_2$, ethanol/ $\text{NH}_4(\text{SO}_4)_2$, glucose/D-alanine and oleate/ $\text{NH}_4(\text{SO}_4)_2$ as carbon and nitrogen sources. Cells were grown for 2 and 4 days at 30 °C. $\Delta pyc1$ cells show a strong decrease in growth on glucose/D-alanine, but not under the other growth conditions. (b) growth (expressed as $A_{660 \text{ nm}}$) of WT (◆) and $\Delta pyc1$ (■) cells on ethanol/L-aspartate. The WT and $\Delta pyc1$ cells grow similarly on this nitrogen source. Cultivation of WT (▲) and $\Delta pyc1$ (●) cells on ethanol/D-alanine shows that the growth of the $\Delta pyc1$ strain is reduced. (c) Dao1p activities were determined in crude extracts of WT and $\Delta pyc1$ cells grown on ethanol/D-alanine. The activities in $\Delta pyc1$ cells were reduced to approximately 30% of the activities detected in WT cells. The mean value of two independent experiments is shown.

strongly suggests that in the absence of Pyc1p, normal assembly/activation of Dao1p is significantly disturbed.

Discussion

This paper describes the identification of two DAOs from the yeast *P. pastoris*: Dao1p and Dao2p. The first enzyme prefers D-alanine as a substrate, is a peroxisomal enzyme, and like peroxisomal AO (Ozimek *et al.*, 2003), is dependent on pyruvate carboxylase for activation.

Microbial DAOs from *Fusarium oxysporum*, *Candida parapsilosis* (Gabler *et al.*, 2000) and *C. boidinii* (Yurimoto *et al.*, 2001) have a high specificity toward D-alanine, whereas DAO from *Rhodotorula gracilis* (Gabler *et al.*, 2000), *Trigonopsis variabilis* (Schrader & Andreesen, 1996) and *Verticillium luteoalbum* (Gabler *et al.*, 2000) display a preference toward

D-methionine. *Pichia pastoris* Dao1p was active toward D-alanine, but not toward D-methionine (data not shown). This is consistent with data for *C. boidinii* DAO that is highly homologous with *P. pastoris* DAO. The activity of *C. boidinii* DAO toward D-methionine is also 86% lower relative to D-alanine (Yurimoto *et al.*, 2001). The pH optimum of *P. pastoris* Dao1p (pH = 9.6) is within the range that has been described for DAOs from other sources (Yurimoto *et al.*, 2001). Earlier work suggested that D-amino acids with a deprotonated amino group are better substrates for DAOs (Harris *et al.*, 2001), and this occurs in an environment with a basic pH. The pK value for the α -amino group proton dissociation amounts to 9–9.5 and matches the pH optimum of *P. pastoris* Dao1p. Lower pH optima (e.g. *T. variabilis*) have been explained by the change in the microenvironment upon binding of the substrate (Harris *et al.*, 2001).

Unlike other DAOs, the *P. pastoris* Dao1p enzyme does not contain a peroxisomal targeting signal, but it is nevertheless a bona fide component of the peroxisome matrix. This suggests that Dao1p belongs to the family of non-PTS proteins that require the PTS1 receptor Pex5p for import (Klein *et al.*, 2002; Gunkel *et al.*, 2004). The actual targeting of such proteins is still unclear, but evidence is emerging that it is in fact a conformational signal rather than a linear epitope that is recognized by the receptor. Whether Dao1p is imported via this alternative route still has to be demonstrated.

Alternatively, the PTS1 recognizing TPR domain of *P. pastoris* Pex5p could have evolved to recognize a different and species-dependent PTS1 consensus and bind to –AAK, the extreme C-terminus of Dao1p. Koller *et al.* (1999) showed that the C-terminus of acyl CoA oxidase (Pox1p), PKI, which does not fit the PTS1 consensus, directs a reporter protein to peroxisomes. However, Elgersma *et al.* (1996) showed that there is a strong requirement for a hydrophobic residue at the ultimate position of the PTS1. The isoleucine of the Pox1p PTS1 fulfills this requirement, but not the C-terminal lysine of Dao1p. Therefore, it remains questionable whether –AAK could represent a targeting signal for Dao1p.

A possible alternative for the import of Dao1p is that it forms heterooligomers with a PTS1-containing protein. An example of this has been shown recently in higher eukaryotes, where the enzyme Cu/Zn superoxide dismutase is imported into peroxisome, together with its PTS1 carrying interaction partner copper chaperone (Islinger *et al.*, 2009).

The physiological role of DAO in eukaryotes is diverse. It plays a catabolic role in lower eukaryotes that allow the organisms to use D-amino acids as carbon and nitrogen sources (Yurimoto *et al.*, 2000). In the human brain, DAO plays a regulatory role and controls the levels of the neuromodulator D-serine. Of interest is the implication of DAO in the pathophysiology of schizophrenia (reviewed in Verrall *et al.*, 2009). A genetic association was observed between the mammal-specific gene G72 and schizophrenia. Yeast two-hybrid studies revealed an interaction between G72 and DAO, and *in vitro* experiments showed an increased enzyme activity of DAO in the presence of G72. However, some unsolved issues remain, such as the different intracellular localizations of G72 and DAO (Chumakov *et al.*, 2002).

Surprisingly, *P. pastoris* cells deficient of the anaplerotic enzyme Pyc1p showed a growth defect in media containing D-alanine as the sole nitrogen source. This defect was specific for D-alanine and due to a reduced enzyme activity of Dao1p. These data lead to the hypothesis that Pyc1p is involved in the activation of *P. pastoris* Dao1p. Pyc1p is also involved in the assembly/activation of peroxisomal AO of methylotrophic yeast species (Ozimek *et al.*, 2003). Binding of FAD is crucial for import of AO (Evers *et al.*, 1996), and in

Apvc1 cells, AO is mislocalized to the cytosol in its monomeric form and lacks FAD (Ozimek *et al.*, 2003). This raised the hypothesis that Pyc1p aids binding of the FAD cofactor to AO monomers, the step required for oligomerization of AO. PpDao1p is similar to AO in that both are peroxisomal flavo-dependent oxidoreductases and bind FAD via the Rossmann fold. The PTS1 of AO is redundant for import and –like Daop1– AO belongs to the class of non-PTS proteins. Apparently, Pyc1p has acquired a second function in methylotrophic yeast to aid the assembly of peroxisomal flavo-proteins.

This phenomenon, termed ‘moonlighting,’ is not restricted to the assembly of peroxisomal proteins, but is observed in many cell functions (Gancedo & Flores, 2008). Multifunctional proteins that have not originated from gene fusion or differential splicing fall into this category. Examples from *S. cerevisiae* are enolase, which catalyzes the interconversion of 2-phosphoglycerate and phosphoenolpyruvate and has a second function in vacuolar protein trafficking (Decker & Wickner, 2006), or Arg83, which is a transcriptional regulator and inositol phosphokinase (Saiardi *et al.*, 1999; Odom *et al.*, 2000). Studies to unravel the molecular principles of the function of Pycp1 in the activation of Dao1p and AO are ongoing.

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